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The Role of RNF4 in Stripping Transcriptionally-Active Huntingtin

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelors of Science in Biology from
The College of William and Mary

by

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Williamsburg, VA
December 8, 2017

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ABSTRACT

Altered gene expression is a hallmark of neurodegenerative disorders such as Huntington's disease. The causative agent of Huntington's is a mutant, aggregation-prone fragment of huntingtin (htt) that aggregates in the cytosol and nuclei of neuronal cells. The severity and onset of Huntington's is correlated with the length of a polyglutamine (Q) tract encoded in the N-terminus of htt. Little is known about the nuclear accumulation of polyQ expanded htt, how it interacts with chromatin, and dysregulates transcriptional activity. In this thesis, we investigate the effect of nuclear localized htt on reporter gene activation and demonstrate that the human SUMO-targeted ubiquitin ligase RNF4 reduces htt's transcriptional activity in a tissue culture model of Huntington's disease. Additionally, we tested the capacity of RNF4 truncations to modulate htt expression – RING and SIM deletions of RNF4 were found to have enhanced inhibitory function compared to full length RNF4, and these unexpected findings are discussed. In summary, our results provide novel insights into the nuclear functions of htt and suggest a model for the functional interaction with SUMO-targeted ubiquitin ligases.

INTRODUCTION

Huntington's Disease

Protein misfolding is a major factor of cellular stress and can result in protein aggregation and the disruption of vital cellular processes. Proteins misfold and aggregate due to proteotoxic stressors including mutations, environmental factors such as pH or oxidative damage, and senescence. Protein aggregation creates a host of problems and has been implicated in the pathology of neurodegenerative disorders such as Alzheimer's, Lou Gehrig's, Parkinson's and Huntington's disease. In the case of Huntington's disease, a region of the *huntingtin* gene on

chromosome IV that encodes a series of glutamine (Q) repeats is expanded, resulting in a harmful, aggregation-prone polyQ tract in the expressed protein. While this gene is vital to cells during development and 17-28 Q repeats are normally present in the huntingtin protein (htt), patients with Huntington's often exhibit upward of 36 Q residues in htt. Due to the high GC content and polymerase slippage, polyQ-encoding tracts that include 29-35 Q are thought to be genomically unstable and prone to changes in length during replication; the overwhelming majority of changes to these unstable glutamine tracts result in elongation of the polyQ tract and propagation of Huntington's disease [Roos 2010]. Patients with trinucleotide repeat disorders including Huntington's experience genetic anticipation, as successive generations have earlier onset and severity of Huntington's due to a gradual generational lengthening polyQ region [Wells 1996]. The length of the polyQ tract is inversely correlated to age of onset of Huntington's disease, with juvenile cases of Huntington's often presenting with polyQ tracts exceeding 55Q and mild, late-onset cases containing between than 30 and 40Q [Roos 2010].

Huntington's disease is inherited in an autosomal dominant manner, as the aggregation-prone form of htt has a dominant negative effect [Hooper 2010]. Homozygosity of disease-causing alleles is very rare due to the infrequent mating of heterozygotes, but there is evidence that homozygotes do not present significantly different symptoms, severity, or clinical course of Huntington's disease [Wexler 1987]. While there are no current treatments for the root cause of the disease, patients are treated for their resulting symptoms. Huntington's appears as deterioration of motor function, neurological processing, and psychiatric state [Roos 2010]. Impaired motor control presents as chorea (involuntary movement) and is often treated with tetrabenazine- a monoamine transport inhibitor or antipsychotic drugs to treat neuropathological symptoms [Paleacu 2007]. Dementia, impaired spatial reasoning, and regressing language processing also

result from the basal ganglia neural atrophy seen in Huntington's disease, and is treated through psychotherapy and speech therapy. Finally, multiple psychiatric changes including depression, anxiety, apathy, irritation, and psychosis are frequently seen in Huntington's patients and treated with antidepressants, antipsychotic drugs, lifestyle changes, and counseling to counteract their elevated suicide risk [Mayo Clinic 2017]. While these treatments may improve quality of life, none of them affect the abnormal folding of huntingtin and its associated functions.

MRI scans and post-mortem analysis of Huntington's patients reveal that there is neuronal degeneration in the striatum of the basal ganglia, cerebral cortex, thalamus, and cerebellum [Kassubek 2004]. The atrophy in the basal ganglia is the most conserved between Huntington's patients, and readily accounts for impaired motor function and cognition. Mutant htt is thought to induce cell death through autophagy and possibly apoptosis, although the exact mechanisms remain unknown [Hooper 2010].

When the mutant, expanded version of htt is produced, it disrupts many cellular processes through its polyglutamine region. Htt normally encodes a 350 kDa protein that contains a glutamine-rich N terminus followed by a polyproline rich region, which is thought to stabilize protein conformation and make htt soluble [Figure 1]. The N terminus also contains 10 HEAT repeats presumed to be involved in protein interactions and vesicle trafficking [Hooper 2010] [Bano 2011]. Htt contains sites for N-terminal cleavage by caspases (cellular proteases), phosphorylation, and SUMOylation. The polyQ tract is flanked by alpha helices and polyproline helices on its N and C terminal sides, respectively [Kim 2009]. Between these helices, the polyQ region adopts multiple conformations, including helices, random coils, and extended loops. When the polyQ region is expanded in Huntington's, the random coil extends and htt inappropriately interacts with other proteins, especially those with polyQ tracts, to form aggregates [Kim 2009].

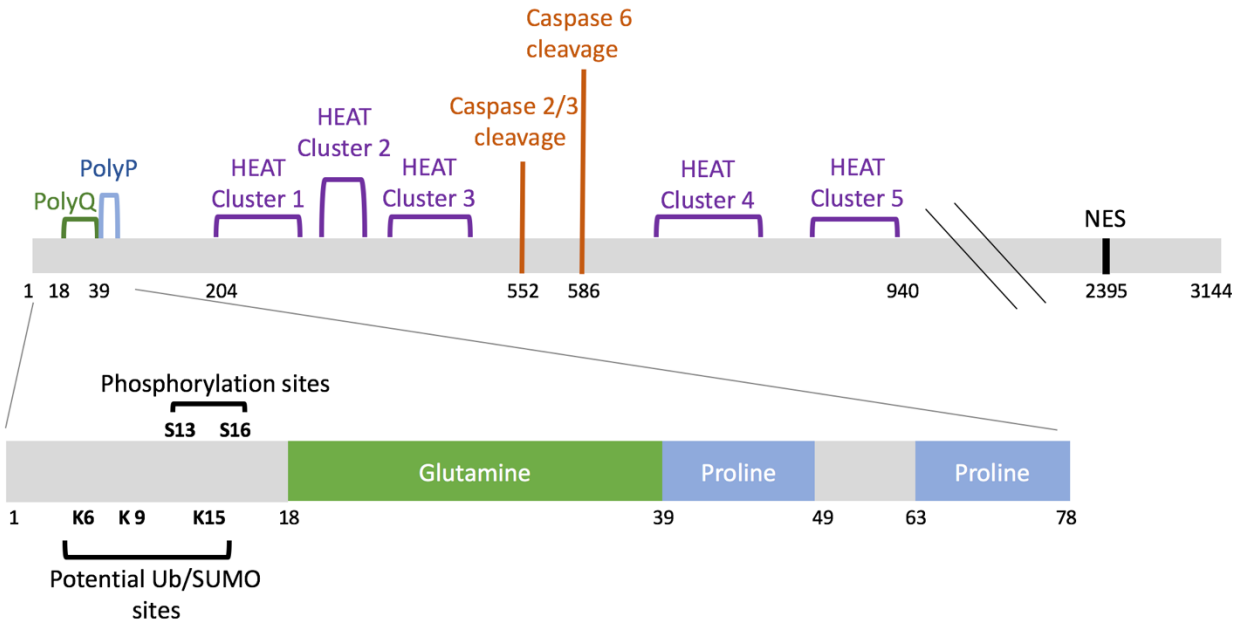


Figure 1. Representation of Huntingtin (htt) and depiction of functional domains. The glutamine repeats located on the N-terminal portion of htt are extended to upwards of 36Q and lead to the neuronal inclusion bodies and atrophy seen in Huntington's patients.

The native function of htt is not entirely clear, though it has been established that htt is an essential protein in neurogenesis, apoptosis, and vesicle trafficking [Hooper 2010]. Htt is found primarily in the cytoplasm, but can localize to the mitochondria, golgi apparatus, endoplasmic reticulum, and importantly the nucleus [Trottier 1995]. Significantly, htt has also been shown to interact with some transcription factors and other regulatory proteins with polyQ tracts [Atanesyan 2012] [Perutz 1994]. Current research has identified significant changes in the transcriptional profile of early-stage Huntington's, notably impairing mitochondrial function and developmental processes [Hagen 2017]. While the polyQ region may serve as a basis for transcription factor interaction, it is also the underlying cause of the neuronal cell aggregates that are characteristic of Huntington's disease.

Protein Aggregation in Huntington's Disease

As mutant htt aggregates accumulate, they have the propensity to form cellular inclusion

bodies. Inclusion bodies are formed when N-terminal fragments of htt interact with each other and recruit other proteins into the growing complex. Glutamine forms hydrogen bonds with other polar residues, and the extended random loop allows for non-specific interactions. PolyQ extended htt can be cleaved by cellular proteases and the fragments of htt appear to be more toxic than the full length version, as cleavage stabilizes the polyQ region and removes the nuclear export signal [Liou 2011]. Inclusion bodies in the cytoplasm disrupt neuronal cell shape and axon function while inclusion bodies in the nucleus are far less understood, but are thought to inappropriately interact with regulatory factors to alter transcription.

Small htt aggregates may not be a problem in and of themselves, but when they recruit essential regulatory factors or cell maintenance proteins into the complex, cell homeostasis is disrupted. For example, mutant htt has been shown to entangle CREB-binding protein (CBP- a protein involved in memory) through glutamine interaction, which eventually leads to neuronal death [Cong 2005]. Interestingly, when the 18Q tract of CBP was removed, the protein was not recruited to the aggregate and neuronal cell death was reduced. Many of the established targets of mutant htt interaction contain polyglutamine regions, suggesting that binding occurs in a glutamine-dependent manner. Aggregates are thought to be a result of the polyQ tract forming new interactions, but other regions of htt also modulate aggregate formation. The proline-rich region that immediately follows the polyQ region modulates both the rate and mode of aggregation, as deleting this region changes the morphology of aggregates while duplicating it decreases the rate of htt aggregation in yeast, a model system used to study hunting aggregates [Arrasate 2012].

Post-Translational Modifications in Cellular Stress

In general, misfolded and aggregating proteins can be targeted for destruction via the ubiquitin-proteasome system (UPS). The covalent attachment of ubiquitin (Ub), a protein around

8.5 kDa, to a target lysine (K) fundamentally alters the target protein's role in the cell. Mono-ubiquitination is thought to serve as a signal, for example leading to endocytosis, membrane trafficking, and altered histone conformation [Sadowski 2010]. Ubiquitin contains lysine at residues 6, 11, 27, 29, 33, 48, and 63, allowing for the formation of poly-ubiquitin chains [Suryadinata 2013]. The location of the poly-ubiquitin attachment results in differential cellular targeting and controls whether the protein is to be degraded. For example, DNA repair mechanisms rely on poly-ubiquitination of proteins through Ub K63 linked chains while the UPS system relies on poly-ubiquitination of targets through attachment of Ub K48 linked chains. The process of attaching ubiquitin to a target protein involves three classes of enzymes. First, an E1 activating enzyme forms a thiol ester bond with Ub in an ATP dependent process and then transfers Ub to an E2 conjugating enzyme. An E3 ubiquitin ligase then binds the E2-Ub complex and facilitates the formation of an isopeptide bond of Ub to the target lysine residue of the substrate. When proteins are modified with Ub K48 chains, the proteasome is able to recognize and subsequently degrade them. The 26s proteasome is made of two subunits, a 19s regulatory particle that unfolds substrates through recognition of poly-ubiquitin signals and a 20s core particle that carry out the proteolytic activity through non-specific cleavage [Nissan 2014]. Proteins need to be partially denatured in the 19s cap to enter the ring-like structure of the proteasome, so protein aggregates that resist unfolding, as seen in Huntington's disease, can halt the UPS machinery [Chang 2015]. However, aggregates may also play a cyto-protective role in Huntington's disease, as cells are isolating mutant htt until the proteasome can degrade it [Liou 2011].

Similar to ubiquitination, proteins can be post-translationally modified with a small ubiquitin-like modifier (SUMO) group, a 12 kDa protein, to modulate their activity, interaction, localization and half-life. SUMO is also attached at lysine residues and the E1 activating, E2

conjugating, and E3 SUMO ligases are comparable to the ubiquitin attachment complex [Gill 2004]. There is a consensus SUMOylation site that has been identified as ΨKXE, where Ψ is a large nonpolar residue. As SUMO attachment is reversible, cells rely on SUMO dynamics to regulate key processes such as the cell cycle, stress response, and sub-cellular localization [Wei 2006; Kerscher 2006]. There have been four different mammalian SUMO homologs identified and they differ slightly in structure and substrate attachment. SUMO-1 shares 18% sequence identity with ubiquitin and is unable to form poly-SUMO chains, as SUMO-1 lacks the consensus motif [Gill 2004]. However, SUMO-2 and SUMO-3 are almost identical in sequence and are known to form poly-SUMO chains through consensus sequence lysine attachment [Hilgarth 2004]. SUMO-4 has only recently been characterized and is thought to be specific to the kidney, although it does not seem to form a conjugatable protein.

Huntington's and other polyglutamine diseases have an interesting connection to SUMOylation. Not only are the levels of SUMOylated proteins increased in these disorders, but SUMO is also thought to stabilize polyQ proteins and make them more soluble [Ueda 2002]. SUMO-1 co-localizes with htt in cells and SUMOylated htt increases neurodegeneration in *Drosophila* [Steffan 2004]. As SUMO and ubiquitin both are attached at lysine residues, SUMOylation may interfere with the UPS system through competition of binding sites on htt [Figure 1]. However, when all the htt lysine residues are mutated to be nonfunctional, neurodegeneration decreases [Steffan 2004]. This indicates that the increased toxicity of SUMOylated htt is more than just in its prevention of ubiquitin attachment.

RNF4, A SUMO-Targeted Ubiquitin Ligase

SUMOylated proteins are not inherently targeted for degradation, but an unusual class of proteins called STUbLs (SUMO-targeted ubiquitin ligases) can send them to the proteasome via

ubiquitin conjugation. These E3 ligases contain SUMO interacting motifs (SIMs) to specifically interact with SUMOylated substrates and a RING domain that dimerizes to recruit Ubiquitin E2 conjugating enzymes [Rojas-Fernandez 2014]. As most STUbLs contain multiple SIMs, they interact preferentially with substrates that have multiple SUMOylated residues [Sriramachandran 2014]. The most well-characterized human STUbL is RNF4 [Figure 2]. RING finger protein 4 (RNF4) contains four tandem SIMs on its N-terminal domain and C-terminal RING domain [Poulsen 2013]. RNF4 is localized to the nucleus and contains a N-terminal bipartite NLS [Kaiser 2003]. Known functions of RNF4 include targeting promyelocytic leukemia protein (PML) nuclear bodies, the kinetochore protein CENP-1, and hypoxia-inducible factors (HIFs) to the proteasome [Sriramachandran 2014]. STUbLs differ in size and structure between species, but remarkably RNF4 can functionally rescue budding yeast and fission yeast strains that lack STUbLs [Cook 2009].

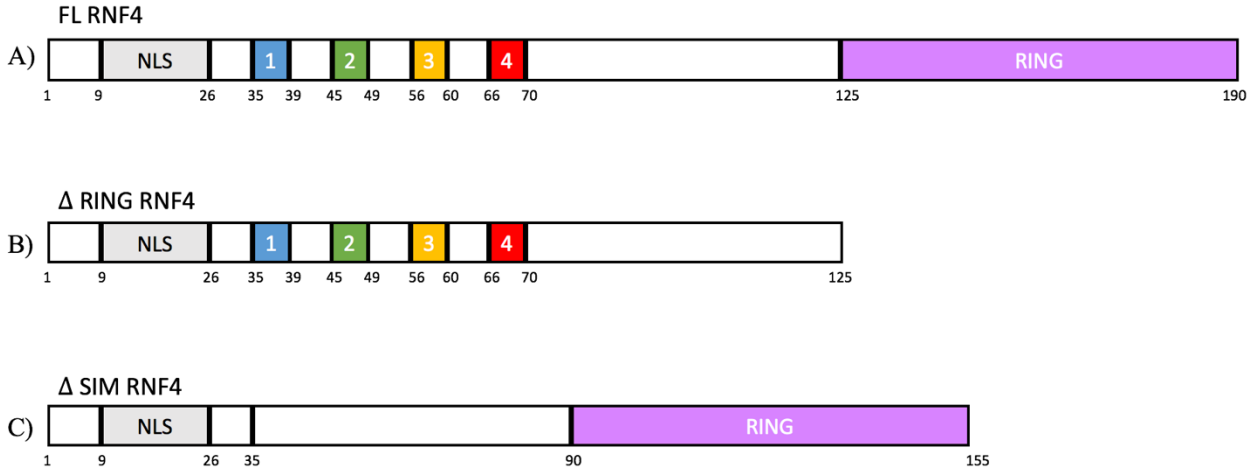


Figure 2. Structural schematic of RING finger protein 4 (RNF4) constructs. (A) Full length (FL) RNF4 contains a N-terminal nuclear localization signal (NLS), four SUMO-Interacting Motifs (SIMs), and a C-terminal RING domain that dimerizes to recruit an E2 conjugating enzyme to ubiquitinylate SUMOylated substrates. (B, C) ΔRING and ΔSIM mutants of RNF4, respectively, are used later in this study to assess what domains of RNF4 are necessary for htt interaction.

While STUbLs have mainly been implicated in the DNA damage response and genome maintenance, the potential to target SUMOylated proteins to the proteasome has far reaching applications in polyglutamine diseases. As the field has shown that htt can become enriched in the nucleus, can become SUMOylated, and is a target of the UPS machinery, STUbLs may represent a potent mechanism for nuclear htt degradation [Guo 2015]. Previous data from our lab have implicated the importance of the yeast STUbLs SLX5 and SLX8 in modulating htt toxicity and aggregate formation [Figure 3]. In yeast strains transfected with human 103Q htt, SLX5 and SLX8 are necessary for cell survival, indicating that these STUbLs play a key role in reducing cellular stress [Figure 3a]. Similarly, an extra copy of SLX5 alters the morphology of aggregates, as htt appears more diffuse in the cell instead of forming inclusion bodies [Figure 3b]. In order to investigate the relationship between SLX5 and htt, yeast two hybrid assays were previously performed in the Kerscher lab. Two hybrid assays are a potent tool to investigate the interaction of two proteins. Briefly, yeast cells are co-transformed with a Gal4-AD (activation domain) fusion of a protein X and a Gal4-BD (DNA binding domain) fusion of a protein Y – interaction of both fusion proteins result in reporter gene activation. Surprisingly, we determined that Gal4-AD fusions of htt activate reporter gene transcription in the absence of a Gal4-BD fusion protein [Figure 4, red arrows]. This significant auto-activation of htt was reduced in the presence of a STUbL Gal4-BD fusion of SLX5 and RNF4 [Figure 4, blue arrows]. These effects provide additional evidence that htt may function as a transcriptional regulator through its polyQ region and that STUbLs are involved in its regulation.

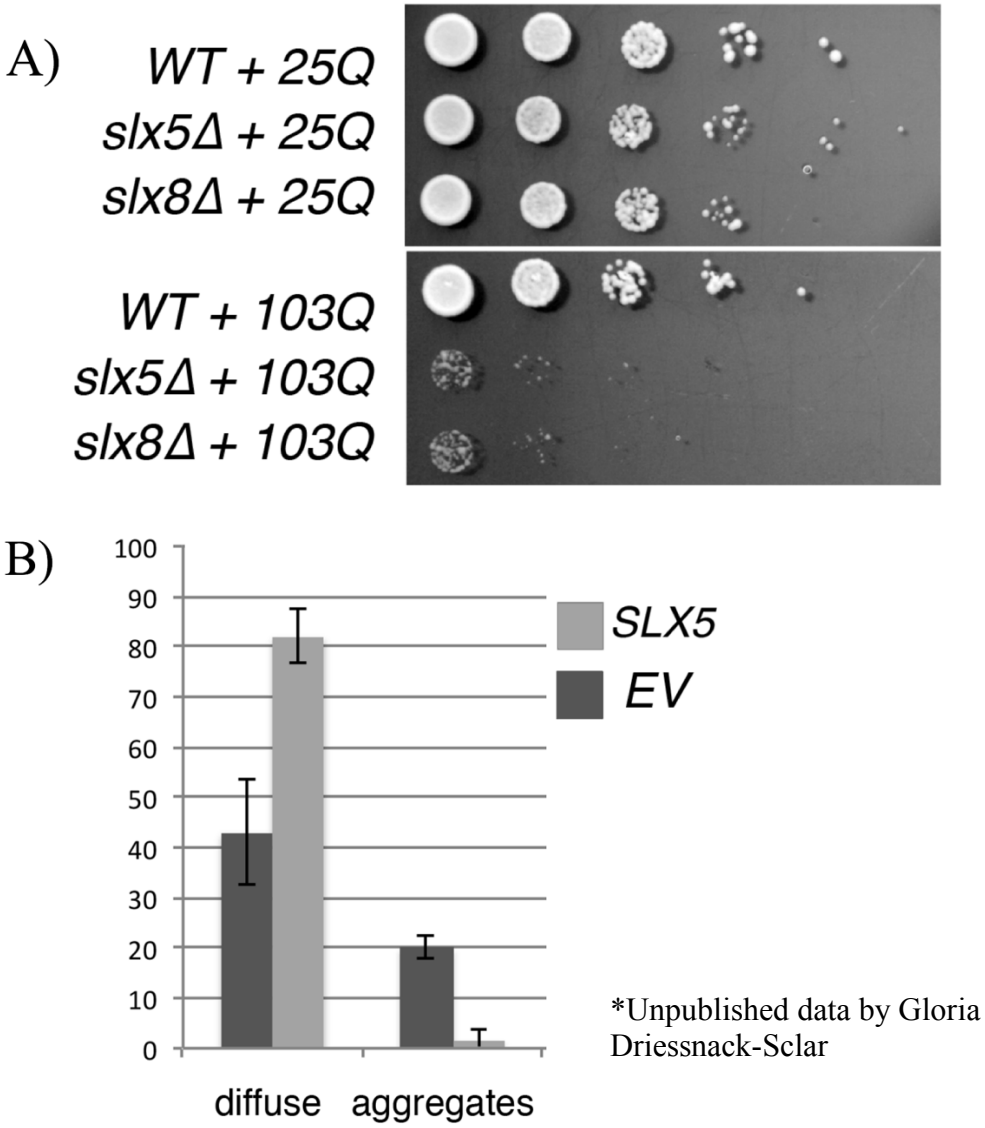
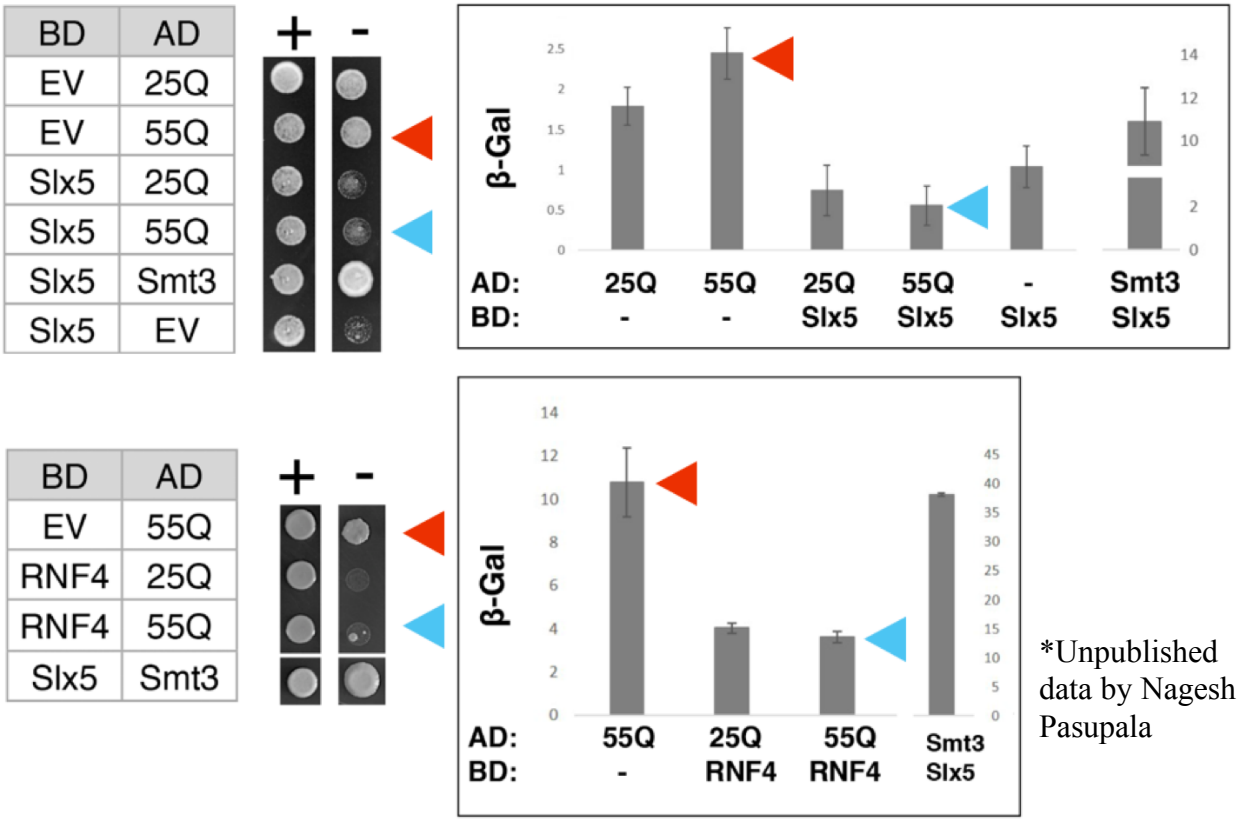


Figure 3. The yeast STUbLs SLX5 and SLX8 are necessary for modulating 103Q htt toxicity and aggregate formation. (A) Yeast strains that contain deletions of SLX5 or SLX8 are still viable with 25Q htt but do not survive in the presence of 103Q htt in a serial dilution spotting assay. (B) WT yeast cells expressing 103Q htt with either a plasmid that contains an extra copy of SLX5 or empty vector (EV). Diffuse staining cells and cells with aggregates were counted and show that SLX5 reduces htt aggregates. These experiments were performed by Gloria Driessnack-Sclar in the Kerscher lab.



*Unpublished data by Nagesh Pasupala

Figure 4. Huntingtin transcriptional auto-activation is reduced with the addition of SLX5 BD and RNF4 BD. Yeast two hybrid assays show that SLX5 and RNF4 decrease htt-dependent HIS3 and lac-Z reporter gene activation. Red arrows show 55Q htt induced reporter gene activation while blue arrows indicate the reduction of htt auto-activation by SLX5 or RNF4. Interaction of Smt3 (yeast homolog of SUMO) and SLX5 represent a positive control. These data were performed by Nagesh Pasupala in the Kerscher lab.

Specific Aims

While yeast is a useful and established model organism for studying specific effects of polyglutamine disorders, conditions such as Huntington's disease rely on the complex dynamics of mammalian cell feedback that cannot always be replicated in non-mammalian models. Therefore, we generated mammalian two hybrid constructs to elucidate the relationship between htt and the human STUbL, RNF4. We hypothesized that there would be significant auto-activation in the mammalian cell model, as seen in the yeast two hybrid assay, and that RNF4 could prevent htt from interacting with DNA.

The specific aims of this thesis are as follows:

1. **To determine whether htt inappropriately activates reporter gene transcription.** We will use two hybrid constructs to assess whether htt auto-activates reporter gene transcription in a variety of mammalian cell lines.
2. **To verify the previously established inhibitory relationship between htt and RNF4 in a mammalian two hybrid assay.** We will assess whether the presence of RNF4 prevents htt from inappropriately activating transcription of a reporter gene in a variety of mammalian cell lines.
3. **To assess the strength of RNF4-mediated inhibition of htt transcriptional activity.** The amount of transcriptional reduction will be analyzed for differential concentrations of RNF4 in an attempt to characterize its potency.
4. **To identify what domains of RNF4 are necessary for htt transcriptional repression.** RNF4 truncations will be tested for their levels of htt inhibition.

MATERIALS AND METHODS

Cell Culture

Thawed tissue culture cells were warmed at 37° C for several minutes before being added to a conical tube containing 5 ml of the appropriate media (detailed below). Cells were spun for 2 minutes at 1000 rpm and the media was aspirated off, leaving the cell pellet intact. The cells were resuspended in 1 ml media and added to a new flask containing 20 ml media. The media was changed 48 hours after starting a new cell line.

HeLa, PC3, PNT2, and LNCaP cells were grown in RPMI media with 10% heat inactivated FBS (ThermoFisher Scientific #10438018) and 1% antifungal/antibiotic (anti/anti) (ThermoFisher Scientific #15240062). HEK 293 cells were grown in DMEM media with 10% heat inactivated FBS and 1% anti-anti. PC12 cells were grown in RPMI media with 10% heat inactivated FBS, 10% horse serum, and 1% anti/anti.

All cell lines were grown at 37° C and 5% CO_2, in a humidified incubator. Cells were split when ~80% confluent and counted using a hemocytometer. Cells at the appropriate confluency were washed using 5 ml dPBS (ThermoFisher Scientific #14190136) and detached with 0.25% trypsin (ThermoFisher Scientific 25200072). Once the cells were detached, they were spun for 2 minutes at 1000 rpm and the media was aspirated off. Cell pellets were then resuspended in 5 ml of the appropriate media and 1 ml was transferred to a new flask with fresh media. To freeze cell samples, cell pellets was resuspended in 1ml media + 10% DMSO, placed inside a styrofoam container, and stored at -80° C.

Mammalian Cell Transfection

Cell were grown to 80% confluency and counted using a hemocytometer. For 96-well plates (Millipore Sigma CLS3799), 5,000 cells in 100 ul media were added to each well while 6-

well plates (Fisher Scientific 07-200-83) received 300,000 cells in 1 ml media in each well. Cells were incubated for 24 hours until 80% confluent.

Earlier stages of the project used Lipofectamine 2000 Reagent while later Lipofectamine 3000 Reagent was used to maximize transfection efficiency and reduce toxicity. In both protocols, 100 ng or 2500 ng of DNA was added to 96-well plate or 6-well plate wells, respectively. For 96-well plate transfections, 0.3 ul of Lipofectamine 3000 reagent and 0.2 ul of p3000 reagent were added to each well. 6-well plate experiments received 3.75 ul of Lipofectamine 3000 and 5 ul of p3000 reagent in each well. Earlier experiments used 0.5 ul and 5ul of Lipofectamine 2000 reagent in 96-well plate and 6-well plate wells, respectively. First, Lipofectamine 2000 or 3000 reagent were diluted in Opti-MEM (ThermoFisher #3195062) and incubated for 10 minutes. The diluted Lipofectamine mixture was then added to the respective DNA vectors diluted in Opti-MEM and p3000 reagent, when appropriate. After a 20-minute incubation, samples were transfected in triplicate for 96-well plate experiments and once for 6-well plate experiments.

The media was replaced five hours after transfection for 96-well plate transfections while the wells were flooded halfway two hours after transfection for 6-well plate transfections and subsequently replaced 24 hours later. For Secreted Alkaline Phosphatase (SEAP) Assays, tissue culture supernatants were collected 48 hours after transfection. Cell extracts were harvested 48 hours after transfection for western blot analysis.

Secreted Alkaline Phosphatase (SEAP) Assay

The Great EscAPe Chemiluminescence kit (Clontech #631737) was used to detect SEAP levels. Twenty-five ul of culture media were obtained and spun for 1 minute at 12,000 rpm to remove cells. The supernatant was transferred to black 96-well plates with clear, flat well bottoms (Corning #353219). Seventy-five ul of 1x dilution buffer was added to each well and the plate was

incubated for 30 minutes at 65° C. The plate was cooled on ice for 3 minutes then equilibrated to room temperature. One hundred ul of SEAP substrate solution was added to each well and the samples were incubated for 30 minutes at room temperature. Chemiluminescent signals were visualized and analyzed using a Li-COR C-Digit Blot Scanner and also autoradiography film.

Cell Extract Preparation

Forty-eight hours after transfection, cells in 6-well plates were washed with dPBS. 300 ul of 1X Cell Lysis Buffer (1X CLB) (Cell Signaling #9803) was added to each well and the samples incubated on ice for 10 minutes with frequent shaking. Cells were scraped to remove any residual adherent cells and the samples were sonicated for 10 seconds at 10% amplitude. The extracts were then clarified for 8 minutes at 15,000 rpm and the supernatant was collected for further analysis.

Western Blot Analysis

Samples were run on a pre-cast NuPAGE 4-12% Bis-Tris Protein Gel (ThermoFisher Scientific NP0321BOX) for 50 minutes at 200 V. Proteins were then transferred to either a polyvinylidene fluoride (PVDF) (ThermoFisher Scientific LC2002) or nitrocellulose membrane (ThermoFisher Scientific #77012) for 25 minutes at 19V. The membrane was blocked in 1xTBS with 4% nonfat dry milk for 1 hours. The respective primary antibody in 1xTBS with 4% nonfat dry milk was incubated with the membrane overnight rocking at 4° C. The membrane was washed three times in 1x TBS containing 0.1% Tween 20 (TBS-T) for 10 minutes before adding the respective HRP-linked secondary antibody in 1xTBS containing 4% nonfat dry milk. The membrane incubated in the secondary antibody for at least 1 hour. Finally, the membrane was washed for at least 30 minutes 1x TBS-T before developing with ECL substrate (Millipore Sigma WBKLS0500). Data shown in this study used a 1:1,000 β-Tubulin Rabbit Polyclonal antibody with a 1:15,000 secondary antibody (Li-Cor 926-42211].

Plasmids used in this study

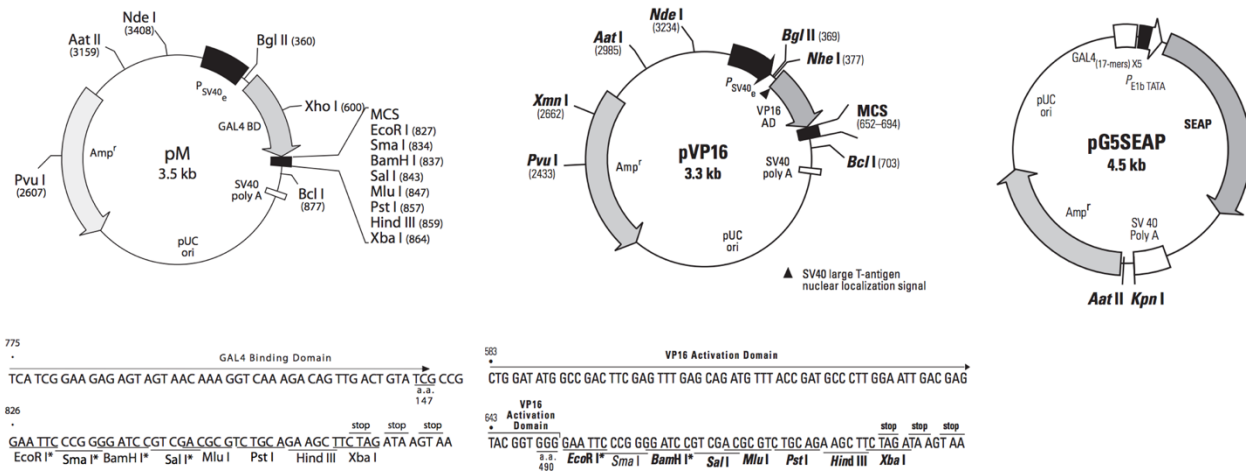


Figure 5. Mammalian two hybrid binding domain (pM), activation domain (pVP16), and reporter gene (pG5SEAP) constructs. RNF4 variants were inserted into the pM-BD vector. 25Q and 55Q htt were inserted into the pVP16-AD vector. The pG5SEAP reporter gene vector allowed for secreted alkaline phosphatase production when activated.

The pVP16 Activation Domain (AD) htt constructs were designed in the Kerscher lab and synthesized by Genewiz (South Plainville, NJ). Different truncations of RNF4 were ligated in the pM Binding Domain (BD) plasmid, as detailed below. The pG5SEAP vector allowed for SEAP transcription when the Gal4 BD was activated, which was subsequently quantified as detailed above [Figure 5]. Colors in the RNF4-BD vectors correlate to the schematic in Figure 2.

pVP16-htt25Q:
MGPKKKRKVAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPH
DSAPYGALDMADFEFEQMFTDALGIDEYGGEFMATLEKLMKAFESLKSFQQQQQQQQ
QQQQQQQQQQQQQQQQQPPPPPPPPPPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAE
EPLHRGSVDASAEASR*

pVP16-htt55Q-AD:
MGPKKKRKVAPPTDVSLGDELHLDGEDVAMAHADALDDFDLDMLGDGDSPGPGFTPH
DSAPYGALDMADFEFEQMFTDALGIDEYGGEFMATLEKLMKAFESLKSFQQQQQQQQ
QQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQQPPPPPPPPP
PPQLPQPPPQAQPLLPQPQPPPPPPPPPPGPAVAEEPLHRGSVDASAEASR*

pM-FL RNF4-BD
MSTRKRRGGAINSRQAQKRTREATSTPEISLEAEPIELVETAGDEIVDLTCESLEPVVVDL
THNDSVVIVDERRRPRRNARRLPQDHADSCVVSSDDEELSRDRDVYVTTHTPRNARDE

GATGLRPSGTVSCPICMDGYSEIVQNGRLIVSTECGHVFCSQCLRDSLKNANTCPTCRKK
INHKRYHPIYI*

pM-ΔR RNF4-BD
MSTRKRRGGAINSRQAQKRTREATSTPEISLEAEPIELVETAGDEIVDLTCESLEPVVVDL
THNDSVVIVDERRRPRRNARRLPQDHADSCVVSSDDEELSRDRDVYVTTHTPRNARDE
GATGLRPSGTVS*

pM-ΔS RNF4-BD
MSTRKRRGGAINSRQAQKRTREATSTPEISLEAEPDERRRPRRNARRLPQDHADSCVVSS
DDEELSRDRDVYVTTHTPRNARDEGATGLRPSGTVSCPICMDGYSEIVQNGRLIVSTECG
HVFCSQCLRDSLKNANTCPTCRKKINHKRYHPIYI*

To obtain pM-RNF4-BD, primers were designed for the *EcoR*1 and *Hind*III sites on the pM BD plasmid. Full length RNF4 (BOK 1282) was purified and amplified using Q5 Hot Start High Fidelity PCR. After verification of the PCR product, RNF4 was cloned and added to competent cells. The cells were then heat-shocked for 45 seconds at 42° C and incubated at for 1 hours at 37° C with shaking. Cells were plated on ampicillin LB plates overnight and the vector was subsequently purified. The pM BD vector and the RNF4 vector were then digested using *EcoR*1 and *Hind*III for 1 hour at 37° C and then the pM BD vector was incubated with calf-intestine phosphatase (CIP) for 30 minutes at 37° C. Both digested vectors were separated on a 1% agarose gel and the correctly digested fragments were extracted from the gel for purification. RNF4 and pM-BD were then ligated together using blunt TA ligase (New England BioLabs M0367S) and then transformed into bacteria. Cells were plated on ampicillin LB plates overnight and the ligated pM-RNF4-BD vector was subsequently purified. Diagnostic digests and sequencing were used to verify the correct construct.

To obtain pM-ΔR RNF4-BD and pM-ΔS RNF4-BD, primers were designed to remove the respective domains. The RING Only primers removed residues 106 to 210 of full length RNF4 and the SIM Only primers removed residues 394 to 570. After size verification on an agarose gel, a Kinase, Ligase, and Dpnl (KLD) (New England BioLabs M0554S) enzyme mixture was used to

ligated the pM-RNF4-BD vector back together. The ligated vectors were then added to competent cells and heat shock for 30 seconds at 42° C. The cells were then incubated for 1 hour at 37° C with shaking and plated on ampicillin LB plates overnight. The purified vectors were then verified with diagnostic digests and sequencing.

Statistical Analysis

Student t-tests were used to analyze statistical significance of SEAP transcriptional levels. A significance level of 0.05 was used to establish significance.

RESULTS

I. *A Mammalian Two Hybrid Assay to investigate the interaction of htt with RNF4*

In this study, we investigated the interaction of htt with the human STUbL protein RNF4 in a mammalian two hybrid system. As described earlier, two hybrid assays are an essential tool to establish if two proteins interact. In the mammalian assay, cells are transfected with three constructs- a Gal4 binding domain (BD) fusion of a protein X, a Gal4 activation domain (AD) fusion of a protein Y, and a reporter gene vector. The Clontech two hybrid system contains a BD construct called pM, an AD construct called pVP16, and the reporter gene is secreted alkaline phosphatase (SEAP) in a construct called pG5SEAP.

In order to assess the interaction of RNF4 and htt, various combinations of AD and BD plasmids were transfected into mammalian cells containing equal amounts of the reporter gene. Gal4 AD htt constructs were synthesized to represent N-terminal htt. 25Q htt AD represents the soluble, non-toxic version of htt while 55Q htt AD represents an aggregation-prone form of htt with moderate toxicity. Cell counts of 25Q and 55Q htt transfected cells were determined to be non-significantly different (data not shown). The positive control condition expressed a fused pM-pVP16 construct that constitutively activated SEAP transcription [Figure 6a]. The negative control condition expressed unfused pM and pVP16 vectors to indicate the level of experimental background of reporter gene activation [Figure 6b]. To measure htt auto-activation, 25Q and 55Q htt AD vectors were co-transfected with EV BD [Figure 6c and 6d]. RNF4's inhibitory effect was assessed by co-transfecting 25Q and 55Q htt AD vectors with RNF4 BD [Figure 6e and 6f]. All transfected cells also expressed the pG5SEAP plasmid, which allowed for quantification of transcriptional activation. Based on the previous yeast two hybrid results in Figure 4, 55Q htt was expected to induce more SEAP transcription than 25Q htt, as 55Q's polyQ tract is modeled after a pathogenic htt fragment.

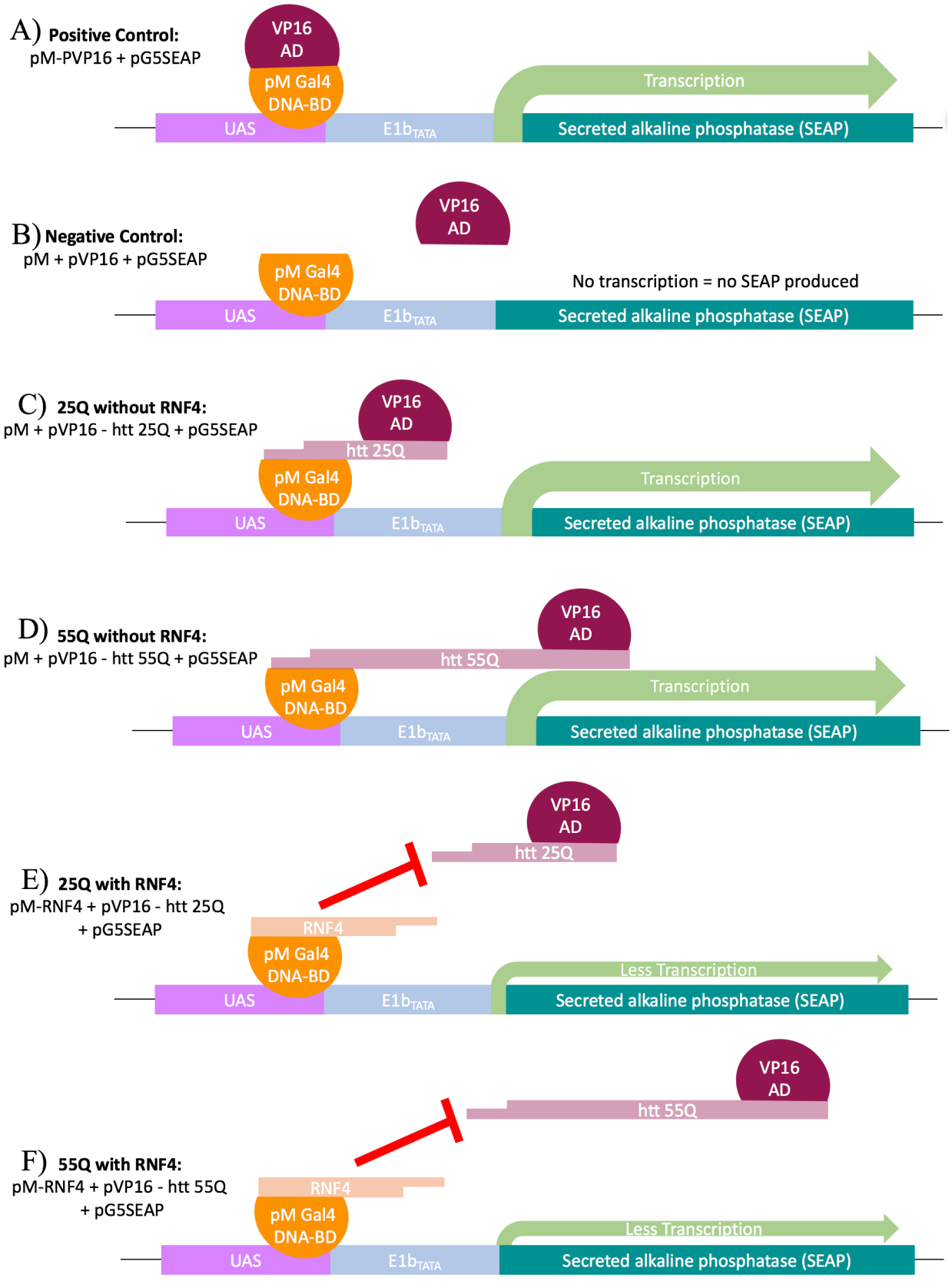

A) Positive Control:
pM-PVP16 + pG5SEAP
VP16 AD
pM Gal4 DNA-BD
UAS
E1b TATA
Transcription
Secreted alkaline phosphatase (SEAP)
B) Negative Control:
pM + pVP16 + pG5SEAP
VP16 AD
pM Gal4 DNA-BD
No transcription = no SEAP produced
UAS
E1b TATA
Secreted alkaline phosphatase (SEAP)
C) 25Q without RNF4:
pM + pVP16 - htt 25Q + pG5SEAP
VP16 AD
htt 25Q
pM Gal4 DNA-BD
Transcription
UAS
E1b TATA
Secreted alkaline phosphatase (SEAP)
D) 55Q without RNF4:
pM + pVP16 - htt 55Q + pG5SEAP
VP16 AD
htt 55Q
pM Gal4 DNA-BD
Transcription
UAS
E1b TATA
Secreted alkaline phosphatase (SEAP)
E) 25Q with RNF4:
pM-RNF4 + pVP16 - htt 25Q + pG5SEAP
VP16 AD
htt 25Q
RNF4
pM Gal4 DNA-BD
Less Transcription
UAS
E1b TATA
Secreted alkaline phosphatase (SEAP)
F) 55Q with RNF4:
pM-RNF4 + pVP16 - htt 55Q + pG5SEAP
VP16 AD
htt 55Q
RNF4
pM Gal4 DNA-BD
Less Transcription
UAS
E1b TATA
Secreted alkaline phosphatase (SEAP)

Figure 6. Mammalian two hybrid constructs. (A) The fused pM-pVP16 construct was a positive control construct that was used to normalize transcriptional levels. (B) The unfused pM and pVP16 constructs indicated experimental background and acted as a negative control. (C, D) Reporter gene auto-activation by 25Q and 55Q htt, respectively, was assessed by transfection of pM-EV and pVP16-htt. (E, F) pM-RNF4 was cotransfected with 25Q and 55Q htt, respectively, to measure RNF4's inhibitory function.

II. *Htt induces reporter gene auto-activation in five diverse mammalian cell lines*

To identify the optimal condition for our mammalian two hybrid htt assays, we tested six different cell lines. The first attempt of the mammalian two hybrid assay was carried out in HeLa cells, a cervical adenocarcinoma line, but due to endogenous placental alkaline phosphatase production, results obtained were inconsistent, prompting us to halt experiments with this cell line. Next, we used three different prostate cell lines to assess the relationship between htt and RNF4: PC3 cells which are derived from a prostate adenocarcinoma, LNCaP cells which are derived from a prostate carcinoma, and PNT2 cells which are derived from a normal prostate epithelium. Human embryonic kidney (HEK293) cells were also used for mammalian two hybrid analysis. Most importantly, PC12 cells, a neuron-like cell line derived from a rat pheochromocytoma, were also subjected to two hybrid analysis.

Huntington's causes the deterioration of neuronal cells which may be partially due to the abnormal transcriptional activity associated with htt. Interestingly, as detailed below, our results suggest that this htt-dependent transcriptional activity can be observed in all cell lines tested [Table 1]. However, due to their neuronal origin, results from the PC12 cell two hybrid should be emphasized and viewed as the most physiologically relevant model of Huntington's disease. Unexpectedly, 25Q htt induced more SEAP transcription than 55Q htt in most cell lines. The notable exception to this trend was observed in neuronally derived PC12 cells; in these cells, 55Q htt exhibited statistically significantly greater transcriptional activation than 25Q htt ($p=0.003$).

Cell Line	Origin	EV-AD	25Q htt-AD	55Q htt-AD
PC3	Prostate adenocarcinoma	0%	86%	75%
LNCaP	Prostate carcinoma	0%	98%	84%
HEK293	Embryonic kidney	0%	83%	68%
PNT2	Prostate epithelium	13%	88%	67%
PC12	Rat pheochromocytoma	26%	88%	100%

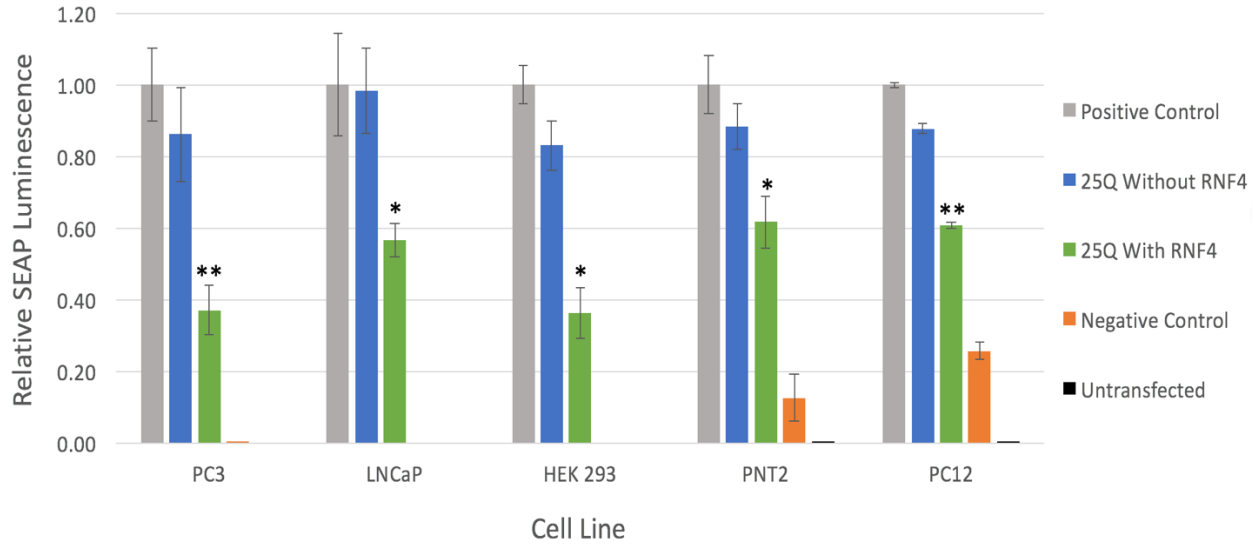
Table 1. Htt auto-activates reporter gene transcription in a host of cell lines. Mammalian two hybrid assays of EV-BD with either EV-AD, 25Q htt- AD, or 55Q htt- AD. Reporter gene auto-activation was measured by 25Q htt-AD and 55Q htt-AD while the experimental background was measured by EV-AD. All values are normalized to the positive control construct's SEAP expression in that cell line (fused pM-pVP16).

III. *RNF4 reduces the transcriptional activity of polyQ constructs*

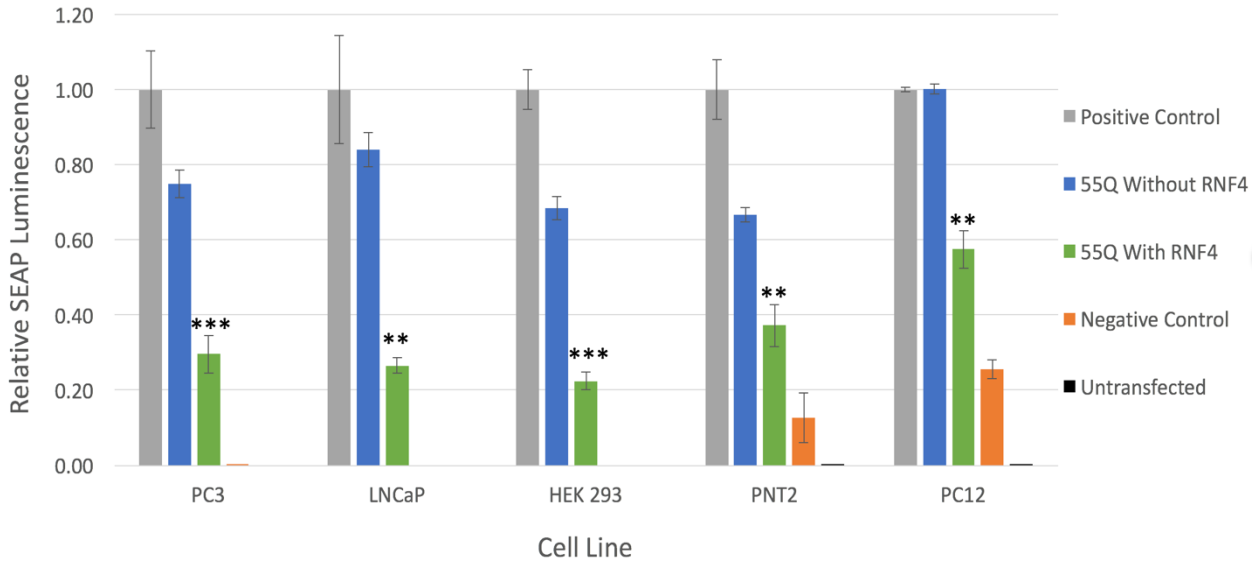
A. *Presence of RNF4 BD reduced htt auto-activation in five mammalian cell lines*

As htt's transcriptional auto-activation may present a major problem for Huntington's patients, this study investigates a RNF4-mediated mechanism to modulate it. Mammalian two hybrid analysis of PC3, LNCaP, HEK293, PNT2, and PC12 cells displayed a significant decrease in the transcriptional activation of 25Q and 55Q htt with the addition of RNF4 BD [Figure 7A and 7B]. In contrast, positive control (fused pM-pVP16) activation, was not reduced by RNF4-BD [Supplemental Figure 1]. The strength of RNF4's inhibitory effect was dependent on the specific cellular environment, but ranged from 30-60% reduction [Table 2]. While the amount of transcriptional reduction varied between cell lines, the overall reductions were statistically significant, indicating that RNF4's inhibitory effect is consistent and replicable. In all cell lines, 55Q htt displayed greater transcriptional reduction (%), suggesting that 55Q htt is more amenable to RNF4's activity [Figure 7C].

A)


1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative SEAP Luminescence
PC3
LNCaP
HEK 293
PNT2
PC12
Cell Line
Positive Control
25Q Without RNF4
25Q With RNF4
Negative Control
Untransfected

B)


1.20
1.00
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0.00
Relative SEAP Luminescence
PC3
LNCaP
HEK 293
PNT2
PC12
Cell Line
Positive Control
55Q Without RNF4
55Q With RNF4
Negative Control
Untransfected

C)

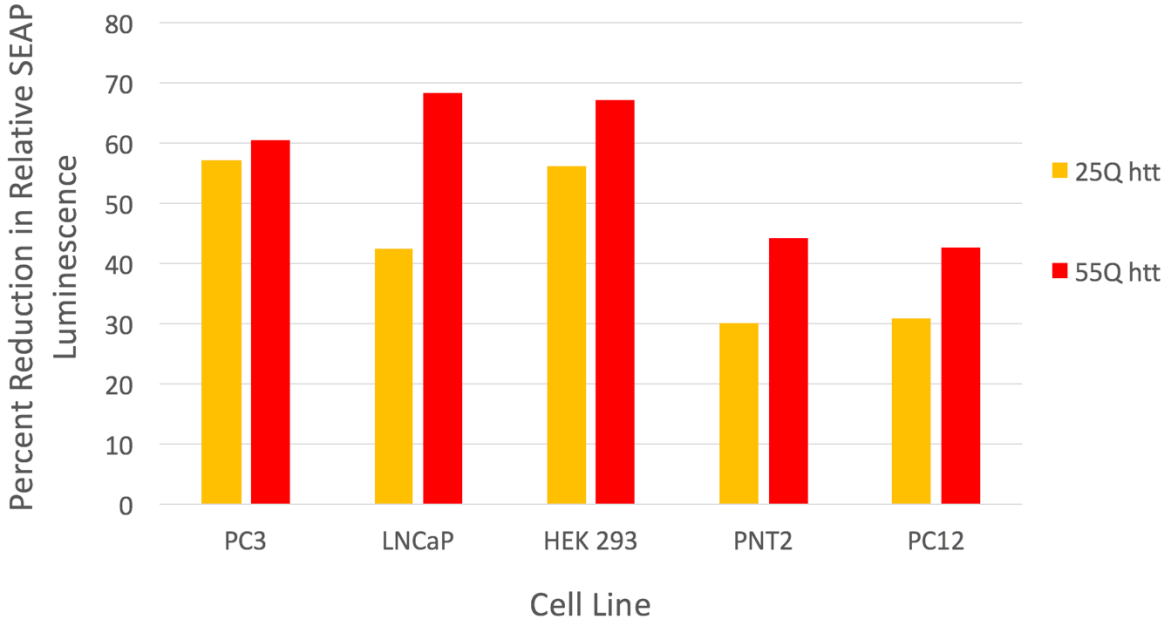


Figure 7. RNF4 reduces the transcriptional activation of htt. Mammalian two hybrid analysis of PC3, LNCaP, HEK 293, PNT2, and PC12 cells [n=3]. (A, B) The conditions described in Figure 6 were tested and normalized to the positive control. Standard deviation is shown by error bars. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (C) Percent reduction in average SEAP luminescence by RNF4 was plotted. Reduction in 25Q and 55Q htt auto-activation by RNF4 is shown in yellow and red, respectively.

AD BD	25Q EV	25Q RNF4	% Reduction	55Q EV	55Q RNF4	% Reduction
PC3	86	37	**57**	75	30	**61**
LNCaP	98	57	**42**	84	27	**68**
HEK293	83	36	**56**	69	23	**67**
PNT2	88	62	**30**	67	37	**44**
PC12	88	60	**31**	100	58	**43**

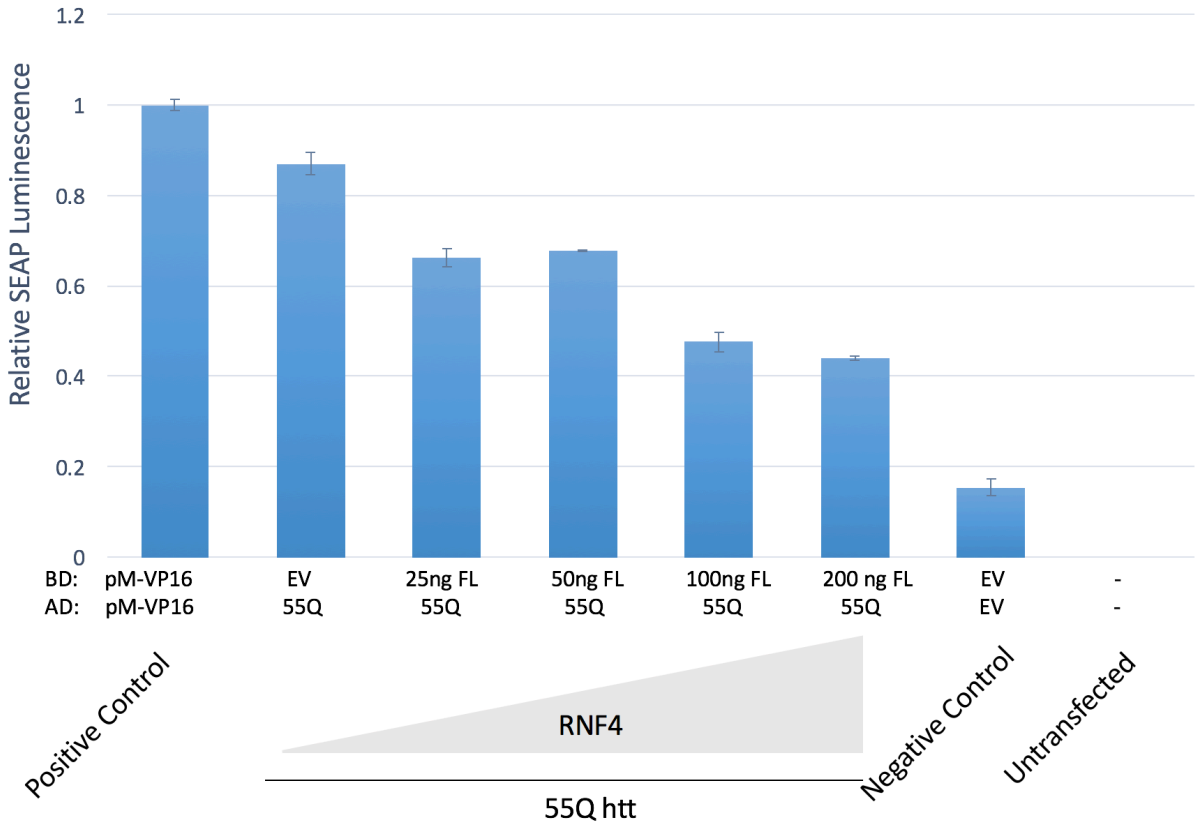
Table 2. Transcriptional reduction of htt auto-activation with RNF4-BD. Difference in relative htt transcriptional activity without and with RNF4 was calculated from the average SEAP luminescence. Values displayed in this table are graphed in Figure 7C.

B. *Levels of RNF4 BD are correlated to the reduction in htt auto-activation*

To test whether RNF4's inhibitory effect was additive, the amount of RNF4 BD was changed while the concentrations of 55Q htt AD and pG5SEAP were held constant in PC3

cells. As 50ng of full length (FL) RNF4 is typically transfected into cells, varying multiples of 50 ng of RNF4 were tested. A general inverse relationship was observed, as increasing FL RNF4 resulted in less transcriptional activity by 55Q htt [Figure 8A]. RNF4's inhibitory function did not linearly correlate with its concentration but more experimentation is necessary to verify the exact relationship between RNF4 concentration and reduction in htt reporter gene activation [Figure 8B]. This dose-dependent relationship could be due to decreasing relative substrate concentrations, as the amount of htt is constant while RNF4 is increasing. One potential possibility is that 55Q htt fails to interact with the reporter plasmid as increasing amounts of RNF4 also increase the likelihood that htt is ubiquitinylated and as other factors are recruited that aid in htt's removal (see discussion).

A)



B)

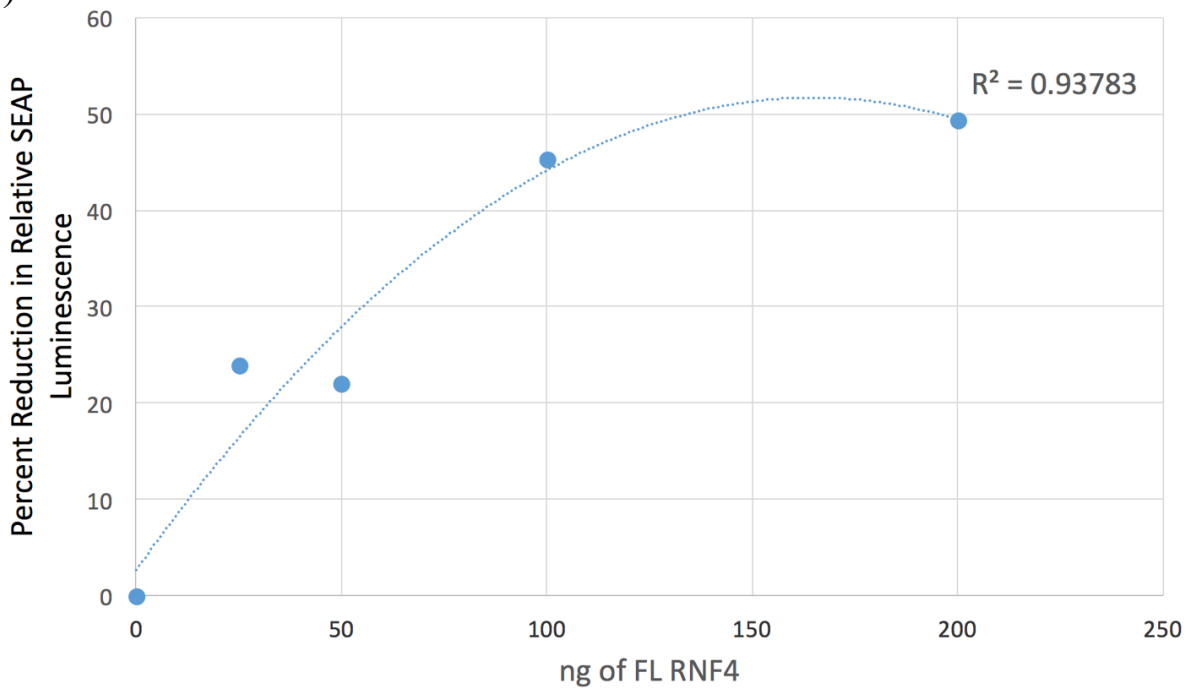


Figure 8. Increasing concentrations of RNF4 lead to a corresponding reduction in Huntingtin's transcriptional activity. Mammalian two hybrid assay of PC3 cells with varying concentrations of full length (FL) RFN4 [n=3]. (A) Positive control, negative control, and untransfected conditions were conducted as described earlier and all conditions were normalized to the positive control. 55Q htt- AD was then co-transfected with either 0, 25, 50, 100, or 200 ng of RNF4- BD and the relative SEAP luminescence was plotted. Standard deviation is shown by error bars. (B) The average reduction in 55Q htt auto-activation was plotted as a function of RNF4 concentration.

C. *An extra copy of non-BD mCherry RNF4 has little to no effect on htt auto-activation*

In order to verify RNF4's inhibitory effect, mCherry RNF4 without a Gal4 BD domain (non-BD) was transfected into PC3 cells along with 55Q htt and pG5SEAP. A reduction in htt-mediated transcriptional activity was observed, although the effect was not statistically significant [Figure 9]. RNF4 is localized to the nucleus, although it primarily is associated with double-stranded DNA breaks [Sriramachandran 2014]. Overexpressing non-BD RNF4 increases the likelihood of RNF4 preventing htt from activating transcription, but RNF4 is not guaranteed to be recruited to that specific transcriptional site of the Gal4 UAS enhancer. The transcriptional

reduction observed with non-BD RNF4 expression is worth noting, but further experimentation is required to verify this effect and establish potential statistical significance.

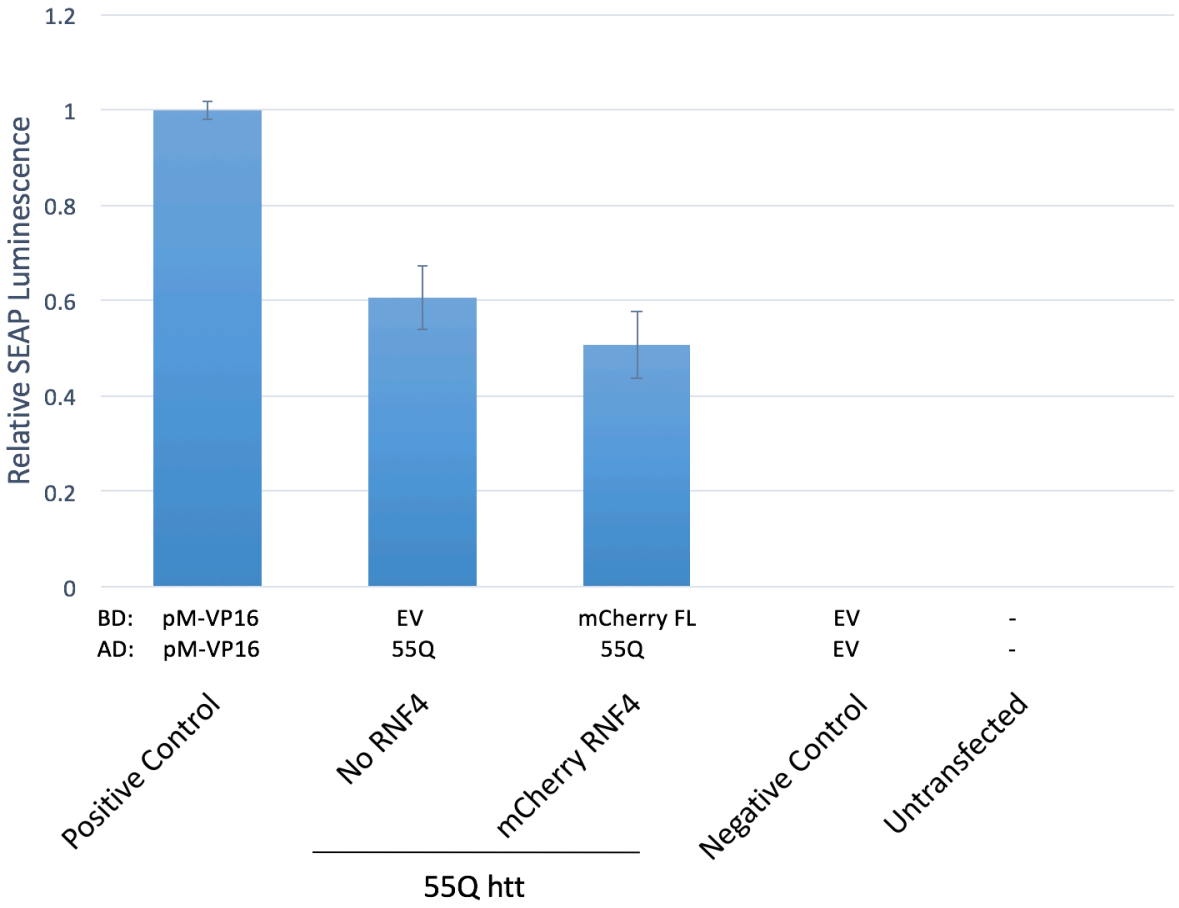


Figure 9. Addition of non-BD RNF4 results in little transcriptional reduction. Mammalian two hybrid analysis of PC3 cells [n=3]. Positive control, negative control, and untransfected conditions were conducted as described earlier and all conditions were normalized to the positive control. 55Q htt- AD was either transfected in alone or with non-BD mCherry RNF4. Standard deviation is shown by error bars.

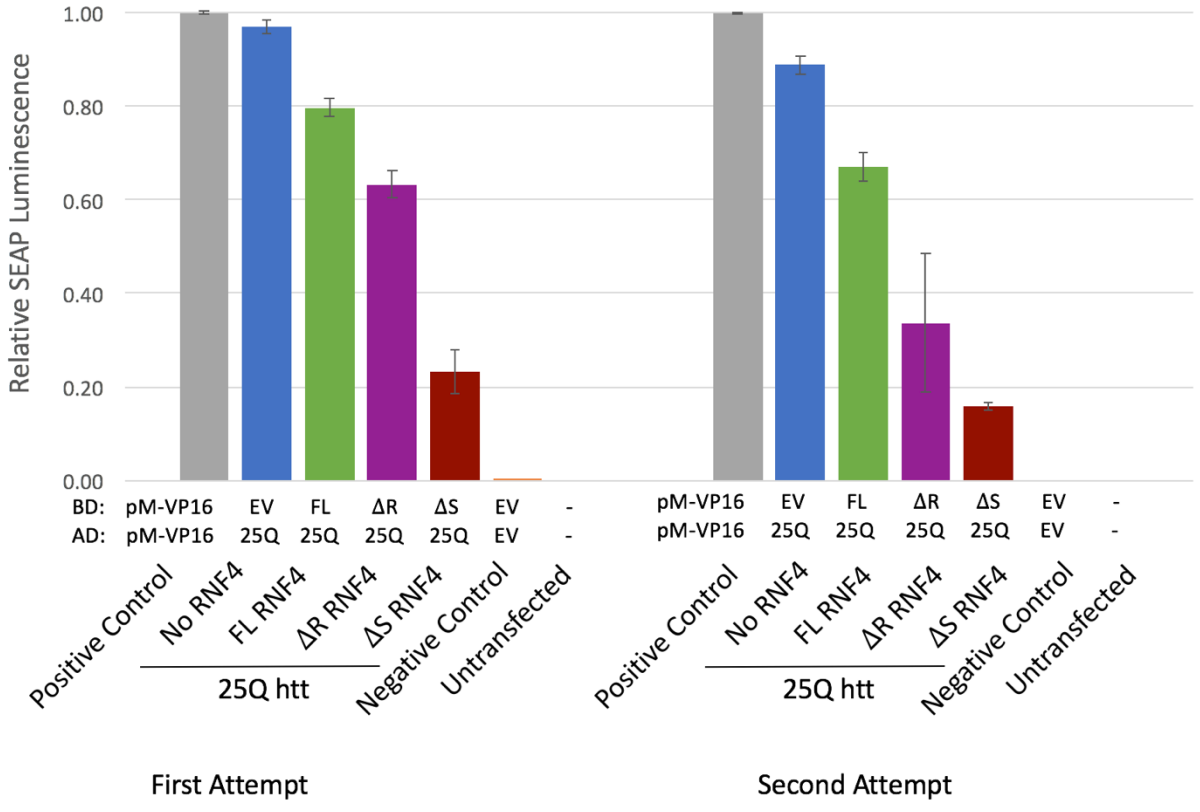
IV. *RNF4 truncations lead to enhanced reduction in Huntingtin's transcriptional Activation*

A. *RING and SIM deletions of RNF4 enhance reduction in 25Q and 55Q htt auto-activation*

To determine the domains of RNF4 that are responsible for its inhibitory function, two RNF4 truncation mutants were generated [Figure 2]. The ΔR mutant lacked the catalytic RING domain while the ΔS mutant had the SUMO-Interacting Motifs (SIMs) of RNF4 removed. It was hypothesized that ΔR RNF4, which retains the SIMs and lacks the RING domain, would inhibit

htt's transcriptional activity less efficiently, as it only contains the recognition sites of RNF4. Similarly, ΔS RNF4 was hypothesized to display a reduced ability similar to FL RNF4, as the RNF4 BD would only lack recognition sites and the RING domain could potentially still dimerize with a native, functional full-length (FL) RNF4 to still carry out the ubiquitinylation function of FL RNF4. Surprisingly, both truncation mutants displayed enhanced ability to reduce 25Q and 55Q htt's auto-activation compared to FL RNF4 [Figure 10]. The ΔS mutant displayed the most robust reduction (76 – 94%) followed by the ΔR mutant (35 – 62%) and the FL RNF4 (18 – 26%). This mammalian two hybrid experiment was repeated five times to verify this effect and two experiments are shown to indicate reproducibility. Cells were grown and transfected in 6-well plates to assess protein levels, and determined to be non-significant between FL, ΔR, and ΔS [Supplemental Figure 2].

A)



B)

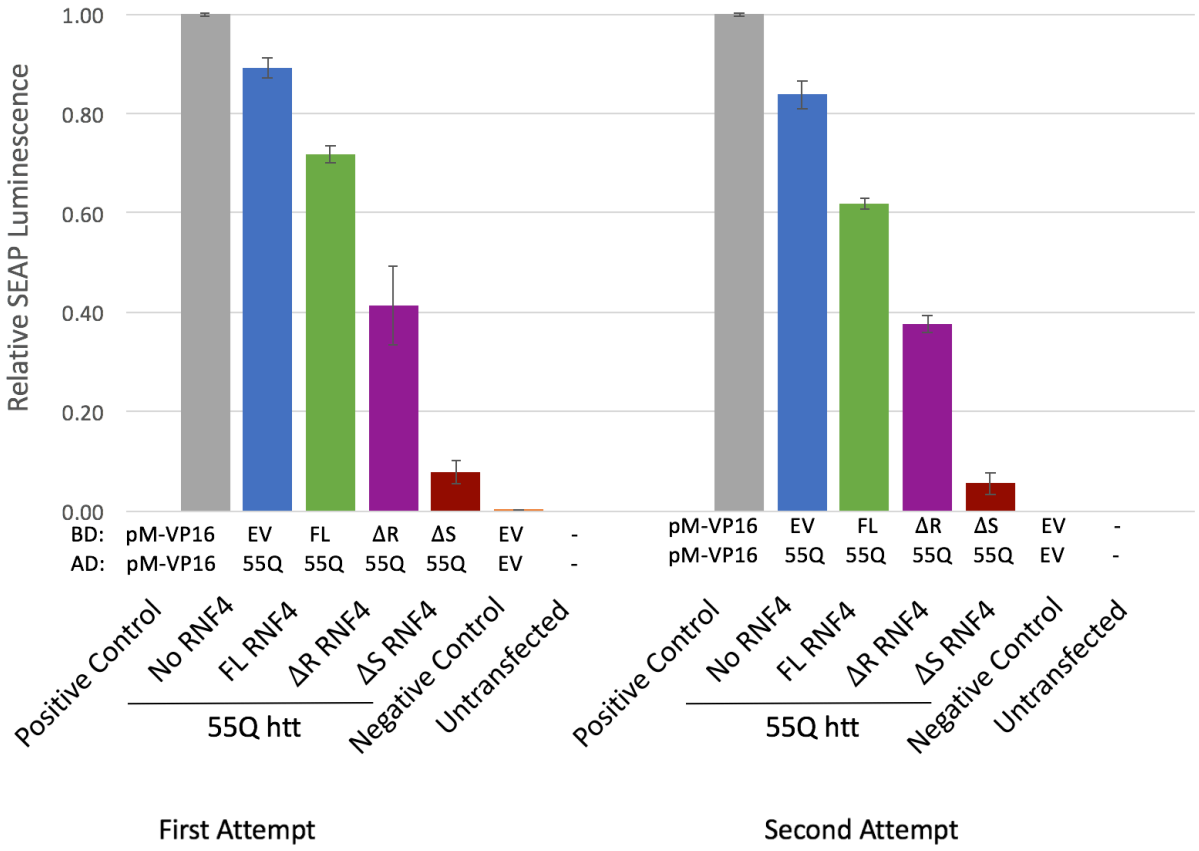


Figure 10. RNF4 truncations lead to enhanced reduction in Huntingtin's transcriptional activation. Mammalian two hybrid analysis of PC3 cells [n=3]. Positive control, negative control, and untransfected conditions were conducted as described earlier and all conditions were normalized to the positive control. 25Q htt- AD (A) or 55Q htt (B) were co-transfected with either EV-BD, full length RNF4 (FL)-BD, a RING deletion mutant of RNF4 (ΔR)-BD, or a SIM deletion mutant of RNF4 (ΔS)-BD. Standard deviation is shown by error bars.

B. *Htt auto-activation is consistently reduced by RNF4 truncations.*

Multiple RNF4 truncation experiments allowed us to statistically evaluate the reduction of htt-mediated SEAP transcription. Since PC3 cells were used in both attempts, the amount of auto-activation present in 25Q htt and 55Q htt were normalized to 0% reduction. The different variants of RNF4 therefore had positive reductions in SEAP. We observed that the percent reduction in SEAP transcription was overall consistent between both experiments, notably so in the 55Q htt results [Tables 3 and 4]. The difference in average transcriptional reduction by RNF4 variants

ranged from 6 – 27% with 25Q htt and 2 – 7% with 55Q htt. This suggests that enhanced reduction by truncations of RNF4 is a real effect and that deleting the RING or the SIMs is possibly making RNF4 less specific in its interactions. If the ΔR and ΔS mutants are non-specifically interacting with a wider range of targets, htt could be blocked from accessing the SEAP transcription site.

AD **BD**	25Q EV	25Q FL RNF4	25Q ΔR RNF4	25Q ΔS RNF4
First Attempt	0%	18%	35%	76%
Second Attempt	0%	25%	62%	82%
Difference	–	7%	27%	6%

Table 3. Transcriptional reduction of htt 25Q by RNF4 truncations. The SEAP luminescence of 25Q htt-AD with EV-BD (auto-activation) was set to 0% and the transcriptional reduction by RNF4 variants were calculated from the data shown in Figure 10A.

AD **BD**	55Q EV	55Q FL RNF4	55Q ΔR RNF4	55Q ΔS RNF4
First Attempt	0%	19%	54%	91%
Second Attempt	0%	26%	55%	94%
Difference	–	7%	2%	2%

Table 4. Transcriptional reduction of htt 55Q by RNF4 truncations. The SEAP luminescence of 55Q htt-AD with EV-BD (auto-activation) was set to 0% and the transcriptional reduction by RNF4 variants were calculated from the data shown in Figure 10B.

The reporter gene auto-activation by htt described in this study supports recent evidence that htt is involved in altering transcriptional profiles. We have shown that this auto-activation can be significantly modulated in five mammalian cell lines transfected with RNF4. Additionally, our data show that SIM and RING deletions of RNF4 are more effective at reducing htt's transcriptional activity, which suggests that the mechanism of reducing htt-mediated transcription by RNF4 is complex and may involve both inter and intra-molecular interactions.

DISCUSSION

The STUbL protein RNF4 plays pivotal genome maintenance roles in mammalian cells but it has only recently been implicated in Huntington's disease [Kerscher 2016]. In this study, we set out to investigate RNF4's role in managing the abnormal transcriptional activity of huntingtin. This research made use of mammalian two hybrid assays to investigate the transcriptional auto-activation of htt and whether RNF4 could prevent htt from interacting with a reporter gene construct. We determined that the presence of RNF4 significantly reduced the transcriptional activation of htt in five different cell lines. Additionally, we found that RNF4 mutants lacking either SIMs or a RING domain retain considerable ability to repress htt-mediated transcriptional activity.

Our results implicate RNF4 in the management of the abnormal transcriptional activity of nuclear htt. In cells that express wildtype huntingtin, this non-expanded htt is not cleaved by proteases and remains cytosolic due to its nuclear export signal. Cells that express polyQ htt have both cytosolic and nuclear aggregates, possibly due to the presence of N-terminal htt fragments that lack a nuclear export signal (NES). Our two hybrid model of Huntington's disease mimics the presence of N-terminal fragments in the nucleus through a pVP16-htt-AD construct which contains an NLS as part of the AD vector. The extensive auto-activation of 25Q and 55Q htt confirms previous observations that htt can interact with DNA and transcription factors, likely through its poly-glutamine tract. The addition of pM-RNF4-BD significantly reduces this inappropriate transcriptional activation and offers a tentative pathway for the removal of nuclear htt. Our current model of RNF4-mediated htt removal and degradation is detailed in Figure 11. In this model, N-terminal fragments of mutated htt form and enter the nucleus after protease-mediated fragmentation. Next, transcription factors and other regulatory proteins interact with htt fragments

via glutamine-mediated interactions. These polyglutamine-mediated complexes disrupt normal transcriptional regulation, as transcription factors are inappropriately recruited to the site of htt-DNA association. The exact transcriptional changes remain unknown and research into this area will clarify how Huntington's disease dysregulates transcriptional programs. Htt can then be SUMOylated by an E3 SUMO ligase such as RanBP2 to increase htt's solubility. As SUMOylated htt begins forming nuclear aggregates and interacting with DNA, RNF4 then is recruited to conjugate ubiquitin chains to htt. These poly-ubiquitin tags are then recognized by UFD1L, which has dual SUMO and ubiquitin recognition sites. NPLOC4 is also recruited via interaction with UFD1L. Finally, VCP/p97 mediates translocation to the proteasome and degradation of htt.

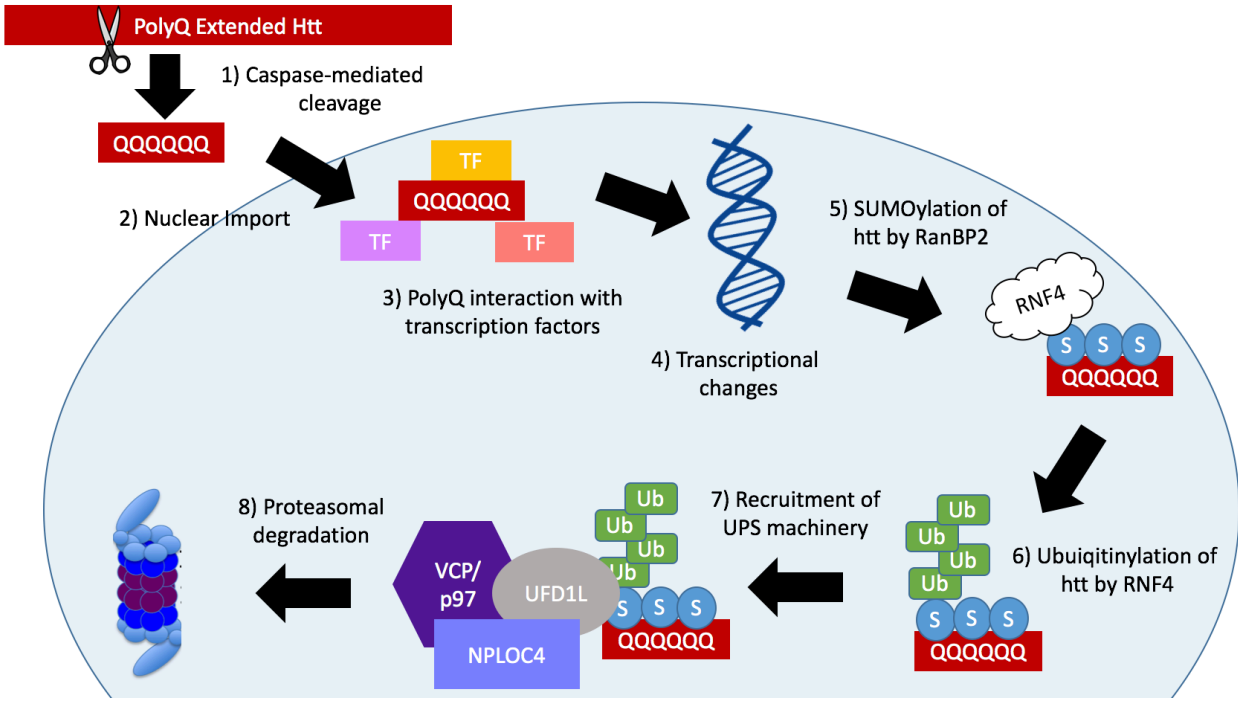


Figure 11. Model of RNF4-mediated degradation of nuclear htt. PolyQ extended, toxic huntingtin (htt) is normally cytosolic, but can be cleaved and enter the nucleus. Htt can then induce transcriptional changes through glutamine-mediated interaction with regulatory proteins. Htt can become SUMOylated by an E3 SUMO ligase and then subsequently ubiquitinylated by RNF4. Finally, the ubiquitin-proteasome system (UPS) can be recruited through dual SUMO-ubiquitin sites and htt can be removed and degraded.

Although the mechanism is unclear, it seems that STUbLs such as RNF4 play a role in managing toxic htt aggregates. Since RNF4 is localized to the nucleus, it is likely that it helps with removing nuclear htt and prevents it from altering the transcriptome. Htt contains three notable lysine motifs in its N-terminus that have been shown to be sites for either Ub or SUMO attachment. While Ub conjugation would target htt to the proteasome, SUMO conjugation actually stabilizes htt and makes it more soluble. When SUMOylated htt accumulates in the nucleus, we are proposing that RNF4 and its associated UPS machinery work to relieve that nuclear stress. This pathway could possibly be manipulated to enhance Huntington's outcomes. By overexpressing RNF4 or inhibiting the E3 SUMO ligase that SUMOylates htt, cells could potentially stabilize their nucleus and prevent further transcriptional changes. RNF4 would be a more ideal target than the E3 SUMO ligase, as RNF4 is more specific and would likely cause less harmful side effects. E3 SUMO ligases are also involved in managing nuclear stress, such as replication fork errors, so inhibiting these ligases would create new problems for cells.

RNF4 is known to be a homodimer, as the dual RING domains recruit an E2 conjugating enzyme to attach ubiquitin to substrates while the SUMO Interacting Motifs (SIMs) function as a regulatory domain to enhance target specificity. As each cell line expresses differential levels of endogenous RNF4, the chance of RNF4 dimerizing to recruit the E2 enzyme and ubiquitinylate substrates also differs between cell lines. Therefore, the extent that RNF4 could repress htt's transcriptional activity was specific to the cellular environment. Even so, the conserved nature of RNF4's inhibitory effect between cell lines was striking.

Synthesizing RNF4 mutants that lacked either their RING or SIM domain allowed for investigation into what domains are necessary for RNF4's inhibitory function of htt. It was hypothesized that the ΔR mutant would exhibit less transcriptional reduction, as it only contains

the regulatory SIMs. However, it was observed that there was greater reduction in SEAP transcription with the ΔR mutant. One possible explanation is that the ΔR mutant nonspecifically interacts with SUMOylated substrates in the cell, including SUMO chains and chromatin. Removing the RING domain decreases RNF4's specificity and possibly alters its structure, allowing the ΔR mutant to interact with a wider range of SUMOylated substrates and preventing htt from activating transcription. It was also hypothesized that removing RNF4's SIM domains would not change its ability to reduce htt's transcriptional activity, as the catalytic RING domain is still present in the ΔS mutant. However, the ΔS mutant again showed enhanced transcriptional reduction. We hypothesize that the ΔS mutant nonspecifically adds ubiquitin to DNA binding proteins, resulting in differential localization. Without the regulatory domain, the RING domain may interact with a greater number of targets, thereby reducing the likelihood that htt could interact with the Gal4-UAS enhancer on the reporter plasmid.

As the ΔS mutant showed the best transcriptional reduction followed by the ΔR and the FL RNF4, it is possible that as RNF4 gains specificity, its interaction with htt is increased. The ΔR and ΔS mutants both may exhibit a wider range of targets, which could prevent htt from interacting with DNA and activating SEAP transcription. FL RNF4 is more specific to htt, as it contains both the catalytic RING and regulatory SIM domains and can properly fold, so FL has fewer targets and the chance of htt transcriptional activation is increased. Since RNF4 only adds ubiquitin to SUMOylated substrates, we would still expect some levels of htt-DNA interaction if htt is not SUMOylated at the time of the experiment. Ultimately, we do not definitively know why the RNF4 truncations displayed enhanced reduction abilities and there are plans to investigate further into these relationships. Truncations are non-physiological and may be incorrectly folded or damaged. To account for these potential structural differences, point mutations should be made to render

either the SIMs or the RING nonfunctional and the mammalian two hybrid assay should be performed with these new constructs. Nevertheless, full-length RNF4 has a potent role in reducing huntingtin's transcriptional activity and its domain functionality, while unexplored, contributes to its inhibitory role.

The limitations of this study necessitate further research into RNF4's role in Huntington's disease. The neuronal PC12 cell line was only obtained late into the project, so many of the experiments were performed in PC3 cells, which had the best transfection efficiency. Future work on this project should replicate results in PC12 cells to verify that the results are conserved in a neuronal environment. It was also found that 25Q htt induced more transcriptional auto-activation than 55Q htt in most cell lines, which is contrary to the known correlation between polyQ tract length and toxicity. However, this phenomenon has been observed in other Huntington's studies, including one publication that found no statistical difference between 20Q and 86Q htt reporter gene auto-activation in HEK293 cells [Atanesyan 2012]. As 55Q htt is more aggregation-prone, it is also possible that our 55Q htt constructs aggregated in the nucleus while 25Q htt was free to interact with our reporter gene transcription site. Relative protein levels also need to be controlled for before concluding on the effectiveness of our constructs. Many attempts were made to quantify BD and AD protein levels but either our cell extract protocol or antibodies were not successful, so we could not make precise conclusions regarding the amount of BD and AD constructs the mammalian cells produced following transfection.

Many applications of this project remain unexplored. Future plans include knocking out RNF4 with either CRISPR-Cas9 or dsRNA to observe cellular effects of htt-mediated transcriptional activity. First, a mammalian two hybrid assay would be performed in the RNF4 knockout and WT cells to determine whether native amounts of RNF4 affect htt's transcriptional

activation. Next, the differential transcriptional profile of the knockout and the WT would be determined using ChIP-Seq of htt. If we can determine what genes htt is prone to interact with, we can better understand Huntington's disease and how the cellular environment is altered. Huntington's disease presents a significant problem in our population, as there is no current treatment that targets its underlying cause. This study opens up possibilities for how cells manage nuclear htt and resist transcriptional changes through the manipulation of the SUMO-targeted ubiquitin ligase, RNF4.

SUPPLEMENTAL FIGURES

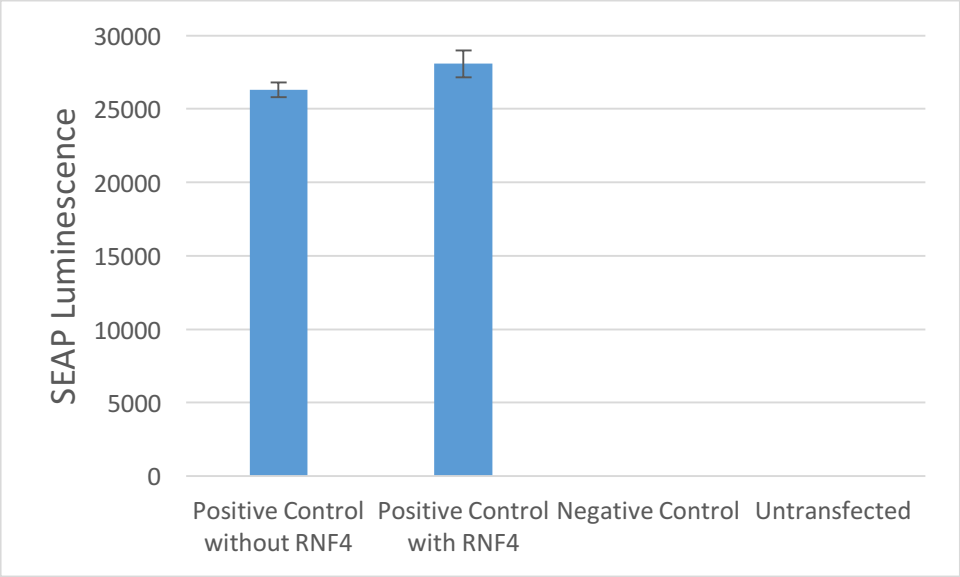


Figure S1. RNF4 does not reduce positive control SEAP transcription. Mammalian two hybrid analysis of PC3 cells. Positive control, negative control, and untransfected conditions were conducted as described earlier. In the experimental condition, the fused pM-pVP16 positive control vector was co-transcribed with RNF4-BD and down to have non-significantly different levels of transcription.

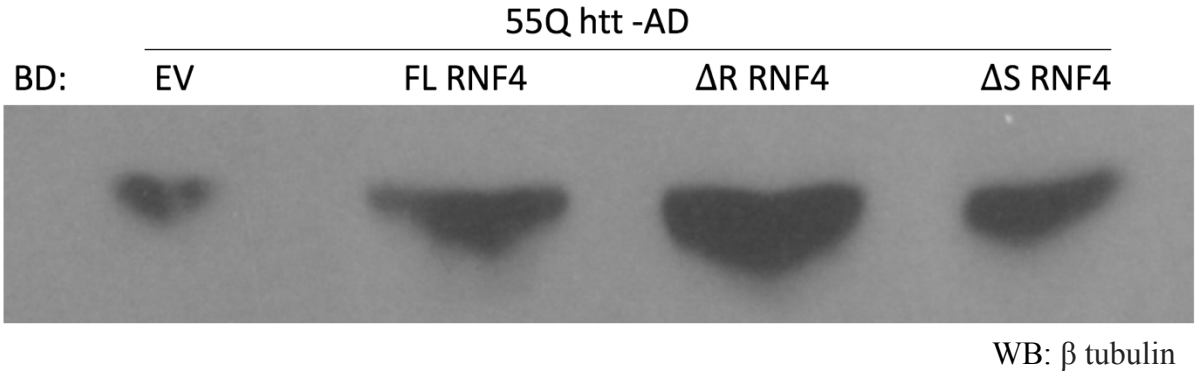


Figure S2. RNF4 BD variants do not affect protein levels. 55Q htt AD was co-transfected with either EV-BD, full length (FL)-BD, ΔR-BD, or ΔS-BD in PC3 cells then β tubulin levels were measured using western blot analysis.

CITATIONS

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